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INVESTIGATIONS INTO THE DISTRIBUTION AND FUNCTION OF NUCLEOBINDIN 1

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Cover: *Nucleobindin 1 is a neuron-specific marker in the rodent brain.*
Maximum intensity projection confocal image of the rat cortex immunohistochemically stained for NUCB1 (green), neuronal marker NeuN (red) and glial marker GFAP (orange), with nuclei counterstained with DAPI (blue).

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Investigations into the distribution and function of nucleobindin 1
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The nucleobindin proteins (NUCB1 and NUCB2) are both characterised by DNA- and calcium-binding motifs. The functionality of these motifs has been demonstrated, but has not yet led to the identification of a role for either protein. The vast majority of literature on NUCB2 has somewhat overlooked these motifs by pursuing the idea that the protein is cleaved to yield a secretory N-terminal peptide fragment. These studies have implicated NUCB2 in a range of physiological processes and diseases, but no mechanism of action has yet been suggested and, furthermore, the endogenous existence of the peptide fragment remains controversial. Meanwhile, investigations into NUCB1 have proposed a number of interaction partners, but have also been unable to define a precise action for the protein.

In this thesis we set out to investigate the distribution and function of NUCB1. Exploring the expression of the protein in the nervous and endocrine systems, we found that NUCB1 exists in a wide array of tissues, but appears to be specifically expressed in secretory cells. This pattern was consistent across the nervous system, where the protein was found in all neuronal populations examined, but absent from glial cells. Similarly, within the endocrine tissues NUCB1 appeared to be restricted to the hormone producing cells, for example the islets of Langerhans, but was devoid from other cell types, such as the exocrine acinar cells. In the pancreatic beta cells and certain populations in the hypothalamus, NUCB1 and NUCB2 were found to be co-expressed, indicating that, despite the structural similarity between these two proteins, they may perform diverse functions.

In order to explore the function of NUCB1 we first developed an organotypic slice culture model of the hypothalamic tuberoinfundibular dopamine (TIDA) network. The electrophysiological properties of these cells have been well characterised, providing a good platform from which to investigate the impact of modulating NUCB1 expression. Furthermore, like the beta cells, the TIDA neurons express both NUCB1 and NUCB2, making both systems useful for studying possible complementary or competing activities of these related proteins. Comparison of the TIDA neurons in organotypic cultures and acute slices revealed that the electrophysiological and morphological properties of these cells are largely unaffected by the culture process, suggesting that the organotypic slices provide a reliable model for functional investigations.

Employing both the organotypic TIDA and isolated pancreatic islet models, we explored the role of NUCB1 in secretory cells. Although altering the expression of NUCB1 was not found to impact cell transcription or calcium handling in the islets, we discovered that knockdown of NUCB1 in the TIDA neurons reduced the frequency of excitatory and inhibitory post synaptic currents. This change could not be attributed to a difference in cell morphology, indicating that NUCB1 may be involved in cell signal transmission.

The work presented in this thesis demonstrates that NUCB1 is broadly expressed in secretory cells and suggests that it may contribute to the signalling responses of these cells. Further investigations are required to elucidate what this contribution may be and whether these findings are consistent with other cells expressing NUCB1.

LIST OF SCIENTIFIC PAPERS

I. Tulke S, **Williams P**, Hellysaz A, Ilegems E, Wendel M, Broberger C (2016) Nucleobindin 1 (NUCB1) is a Golgi-resident marker of neurons. *Neuroscience* 314: 179-188

II. **Williams P**, Tulke S, Ilegems E, Berggren PO, Broberger C (2014) Expression of nucleobindin 1 (NUCB1) in pancreatic islets and other endocrine tissues. *Cell Tissue Res* 358:331

III. **Williams P**, Grivet Z, Stagkourakis S, Lagerlof O, Broberger C (2019) A mouse organotypic culture model of the tuberoinfundibular dopamine (TIDA) network. *Manuscript*

IV. **Williams P**, Ilegems E, Berggren PO, Broberger C (2019) The impact of altered nucleobindin 1 (NUCB1) levels on cell signalling in the brain and islets of Langerhans. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

CNS	Central nervous system
DAT	Dopamine transporter
DAPI	4’,6’-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DIV	Days *in vitro*
ELISA	Enzyme-linked immunosorbent assay
EPSC	Excitatory postsynaptic current
GFAP	Glial fibrillary acidic protein
IPSC	Inhibitory postsynaptic current
mRNA	Messenger ribonucleic acid
NUCB1	Nucleobindin 1
NUCB2	Nucleobindin 2
MBP	Myelin basic protein
RIP	Rat insulin promoter
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
shRNA	Short hairpin RNA
TH	Tyrosine hydroxylase
TIDA	Tuberoinfundibular dopamine

1 INTRODUCTION

In the beat of a heart a branch of cell biology was born. One hundred and thirty years since Sydney Ringer's discovery that calcium is integral to the contraction of the heart (Ringer, 1883), the role of this ion as a signalling molecule is well-appreciated – and still being intensively explored. Involved in almost all cellular processes from proliferation to apoptosis, adhesion to motility, gene expression to hormone secretion, the fact that calcium is able to mediate a wide range of effects and processes is largely due to two factors: a steep concentration gradient of the ion inside the cell, and an extensive catalogue of diverse proteins to which it can bind. These proteins can be broadly categorised as either buffers, which bind calcium for the primary purpose of regulating this potent messenger's concentration and distribution within the cell, or sensors, which generally change conformation upon interaction with calcium, enabling them to interact with downstream targets in a signalling cascade (Berridge, 1993; Carafoli et al., 2001; Clapham, 2007; Schwaller, 2010).

The functions of some of these proteins have been extensively studied and are becoming increasingly well understood such as calbindin D-28k, parvalbumin (Baimbridge et al., 1992), troponin-C (Grabarek et al., 1992) and calmodulin (Chin and Means, 2000). Furthermore, through detailed reports of their selective distribution, calcium binding proteins have emerged as valuable tools in the identification of discrete cell populations in the brain (Celio and Heizmann, 1980; Celio, 1990; Andressen et al., 1993). Meanwhile, the family of calcium binding proteins continues to grow and the role of other members, such as nucleobindin 1 (NUCB1), remain to be established.

1.1 NUCLEOBINDIN 1

NUCB1 (also known as Nuc and CALNUC) was first identified as a secreted protein produced by cultured B cells, derived from a mouse model of systemic lupus erythematosus, and proposed to be a growth and differentiation factor involved in the production of anti-DNA antibodies (Kanai and Tanuma, 1992). Initial analysis of the protein structure of NUCB1 revealed a 455 amino acid chain containing an N-terminal hydrophobic sequence, as well as a basic amino acid rich region and a leucine zipper (Miura et al., 1992) – a motif combination commonly associated with transcription factors (Landschulz et al., 1988). The functionality of these motifs was verified through the synthesis of recombinant NUCB1 and associated deletion mutants. Mobility shift assays demonstrated the ability of the recombinant protein to bind to DNA, while deletion of either the basic amino acid rich region or the leucine zipper reduced or abolished this property, respectively (Miura et al., 1992). Later scrutiny of the protein structure uncovered the presence of two EF-hand motifs (Miura et al., 1994; Fig. 1), and deletion of both EF-hands abolished the calcium binding ability of the full length protein. Furthermore, interaction with the ion was reported to have no impact on the ability of NUCB1 to bind DNA (Miura et al., 1994).

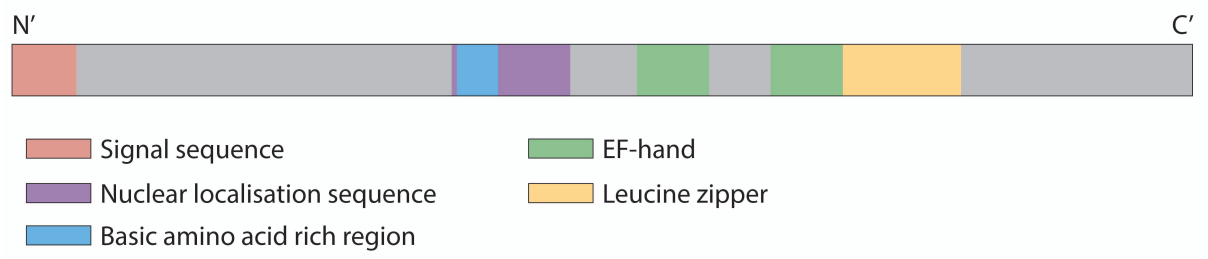

Figure 1: Schematic of the primary structure of NUCB1 showing the location of functional motifs.

This rare phenomenon of a protein containing both DNA- and calcium-binding motifs was also encountered a couple of years later, in a human leukaemia cell line, with the discovery of NEFA – a protein determined to share a high degree of sequence similarity with NUCB1, particularly in the EF-hand and basic amino acid regions (Barnikol-Watanabe et al., 1994), to the extent that it subsequently became known as nucleobindin 2 (NUCB2).

Although both proteins were first detected in cell culture media, the existence of these motifs indicated that the nucleobindins may perform intracellular roles. The first published histological analysis using an antibody against NUCB1 revealed it to be predominantly located within the nucleus in tumour cells from gastric adenocarcinomas (Wang et al., 1994), a finding echoed later in non-Hodgkin's lymphoma cells (Kubota et al., 1998) as well as odontoblasts (Somogyi et al., 2004) and differentiating osteoblasts (Petersson et al., 2004). In addition, the protein structure was found to include a potential nuclear localisation signal (Wendel et al., 1995), providing further support for the prospective DNA-binding function of NUCB1.

The greater wealth of NUCB1 literature to emerge, however, focused on the calcium binding properties of the protein and the search for possible interaction partners. Immunofluorescence and immunoelectron microscopic studies performed in rat and human cell cultures demonstrated that NUCB1 was localised to the *cis*-Golgi network and, furthermore, represented the major calcium-binding protein of the Golgi (Lin et al., 1998). This led to the suggestion that NUCB1 plays an important role in Golgi calcium homeostasis and mediates calcium release from the organelle (Lin et al., 1999). The notion that NUCB1 could essentially play such a fundamental role in cell homeostasis was given further weight by other studies detailing the wide-ranging expression of NUCB1, not only in various organs of the human, including the brain, heart and pancreas (Miura et al., 1996), but also through its apparent evolutionary preservation via the discovery of homologs in diverse species including Drosophila melanogaster, Spodoptera frugiperda and Caenorhabditis elegans (Otte et al., 1999; Kawano et al., 2000; Cuppen et al., 2003).

However, a detailed structural study on the EF-hand region of NUCB1 revealed that calcium binding induces a change in the protein's conformation, exposing a large hydrophobic surface. While not ruling out calcium-buffering activity, this finding raised the prospect of NUCB1 being more likely to function as a calcium sensor (de Alba and Tjandra, 2004). This calcium-induced conformational change was found to mediate the interaction of NUCB1 with the heterotrimeric G protein subunit $G_{\alpha i3}$ (Lin et al., 2000; Garcia-Marcos et al.,

2011), although it has also been proposed that, conversely, binding to the G-protein facilitates the ability of NUCB1 to bind calcium (Kanuru et al., 2009).

In addition to alternative G_α subunits (Mochizuki et al., 1995; Lin et al., 1998; Weiss et al., 2001; Lin et al., 2009), a range of other NUCB1-interacting proteins have been revealed, including cyclooxygenase 1 and 2 (Ballif et al., 1996; Leclerc et al., 2008), the neural growth suppressor necdin (Taniguchi et al., 2000), the breast cancer-associated membrane protein BCMP84 (Adam et al., 2003), the low-density lipoprotein receptor related protein LRP9 (Brodeur et al., 2009) and amyloid polypeptide (Gupta et al., 2012; Bonito-Oliva et al., 2017). Meanwhile, a variety of functions beyond cell calcium homeostasis have been attributed to NUCB1, including downstream regulation of G-protein signalling (Kanuru et al., 2009; Lin et al., 2009; Kapoor et al., 2010), regulating retrograde transport of lysosomal receptors (Larkin et al., 2016) or the unfolded protein response (Tsukumo et al., 2007), modulating levels of amyloid precursor protein and inhibiting fibril formation through chaperone-like activity (Lin et al., 2007; Gupta et al., 2012; Bonito-Oliva et al., 2017; Kanuru and Aradhyam, 2017), serine protease activity (Kanuru et al., 2013), tumorigenesis in colon cancer (Chen et al., 2007) and as a precursor to an insulin secretion-stimulating peptide (Ramesh et al., 2015).

But despite being associated with this broad range of interacting proteins and implicated in a variety of intracellular roles, in both healthy and disease states, no dominant principle regarding the function of NUCB1 has yet emerged.

1.2 NUCLEOBINDIN 2

First reported two years after the discovery of NUCB1, NUCB2 is also characterised by the coexistence of DNA- and calcium-binding regions (Barnikol-Watanabe et al., 1994) and shares 60% sequence similarity with its predecessor (Karabinos et al., 1996). However, NUCB2 received relatively little attention until 2006, when it was proposed to be a precursor protein to a cleaved and secreted peptide, nesfatin-1, which induced satiety when injected into the brain (Oh et al., 2006). This prompted a wave of research into the effects of NUCB2/nesfatin-1, which has since been implicated in a range of physiological functions including food and water intake (Stengel et al., 2011; Yosten et al., 2012), blood glucose homeostasis and insulin secretion (Foo et al., 2010; Nakata et al., 2011; Riva et al., 2011), onset of puberty (Garcia-Galiano et al., 2010; Garcia-Galiano et al., 2012) and sleep (Vas et al., 2013). In conjunction with these studies, the expression of NUCB2 has been reported in a variety of tissues including the pituitary (Foo et al., 2008), gastrointestinal tract (Stengel et al., 2009; Zhang et al., 2010) and the testes (Stengel et al., 2009), while a substantial amount of attention has been focused on its expression in the hypothalamus (Brailoiu et al., 2007; Goebel et al., 2009; Goebel-Stengel et al., 2011; Maejima et al., 2016) and the pancreatic beta cells (Foo et al., 2008; Gonzalez et al., 2009; Stengel et al., 2009; Gonzalez et al., 2011; Nakata et al., 2011; Riva et al., 2011).

To date there remains a lack of evidence for the endogenous cleavage of NUCB2 and subsequent secretion of a nesfatin-1 peptide (Foo et al., 2008; Stengel et al., 2009), and to date no receptor for it has been identified. It thus remains possible, especially in light of the functional domains present in the protein, that NUCB2 may also perform an intracellular role.

If this is the case, given the high degree of similarity between NUCB1 and NUCB2, it remains to be determined whether the two proteins perform diverse or complementary functions in the same, or mutually exclusive, cell types.

2 AIMS

The work presented in this thesis set out to advance current knowledge of the distribution and function of NUCB1. The investigations were broken down into four separate studies with the following specific aims:

- Determine the distribution of NUCB1 in the rodent central nervous system (CNS). (Study I)

- Identify the expression pattern of NUCB1 within the rodent endocrine system. (Study II)

- Develop an organotypic slice culture model of the dopaminergic neuroendocrine system that can be used to assess the function of NUCB1. (Study III)

- Investigate the role of NUCB1 in the function of pancreatic islets and hypothalamic neurons. (Study IV)

3 METHODS

A detailed description of the methods used for each study contained within this thesis can be found in the respective papers. Considerations on the limitations of techniques employed in the thesis work are addressed with the discussion of results. Presented here is a brief overview of the two model systems used in this work for studies into the function of NUCB1 – the isolated islets of Langerhans and the organotypic slice culture of the tuberoinfundibular dopamine (TIDA) network.

3.1 ISOLATED ISLETS OF LANGERHANS

The islets of Langerhans are small clusters of endocrine cells which are dispersed throughout the pancreas and are primarily responsible for the regulation of blood glucose levels (Langerhans, 1869; von Mering and Minkowski, 1889; Laguesse, 1893). The islets are comprised of five major cell types defined by the hormone they produce and release: glucagon-secreting alpha cells, insulin-secreting beta cells, somatostatin-secreting delta cells, pancreatic polypeptide-secreting PP cells and ghrelin-secreting epsilon cells (Fig. 2). Beta cells comprise the majority of the cells in the islet and high glucose concentrations in the blood prompt them to secrete insulin (Fig. 3), stimulating tissues such as the liver and skeletal muscle to take up glucose from the blood. Alpha cells oppose this effect with the secretion of glucagon, while somatostatin contributes to regulating the secretion of both of these hormones. As well as being highly vascularised (Brunfeldt et al., 1958; Bonner-Weir and Orci, 1982), islets also receive extensive innervation (Smith and Davis, 1983; Ahren, 2000) which modulates their hormone release. Furthermore, paracrine and autocrine signalling provides an additional layer of complexity to the organisation and regulation of islet secretion (Taborsky et al., 1979; Leibiger et al., 2002; Leibiger et al., 2012).

Islet isolation by collagenase digestion was first described in 1965 (Moskalewski, 1965) and, after refinement of the technique to include injection of the collagenase into the pancreas

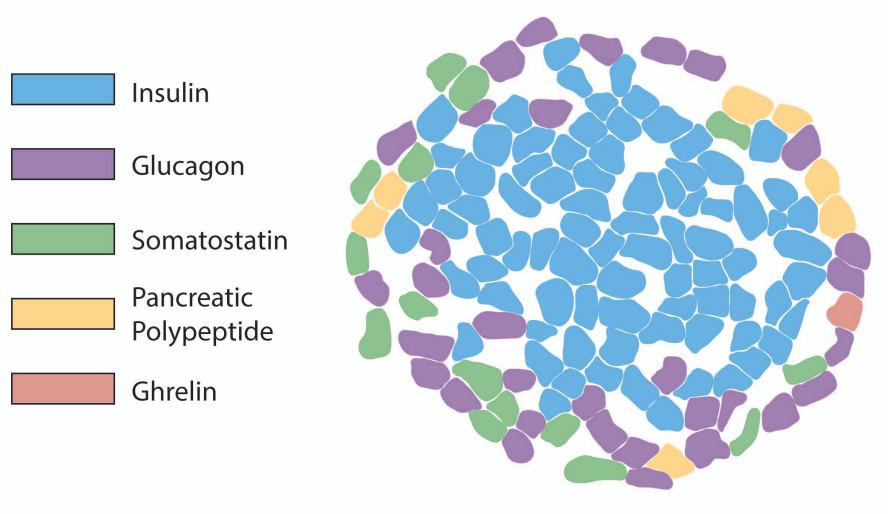

Figure 2: Schematic representation of the proportion and distribution of the five endocrine cell types in the islets of Langerhans.

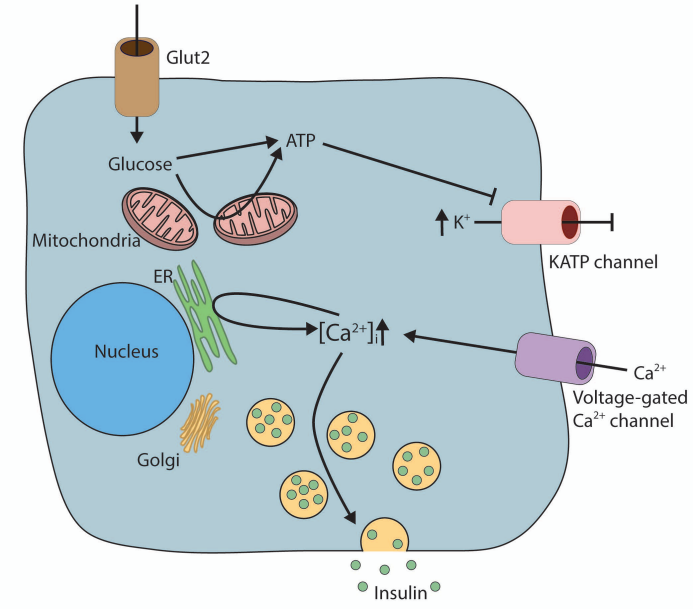

Figure 3: Overview of glucose stimulated insulin secretion in the mouse beta cell. As blood glucose levels rise, it is taken up by the beta cells through Glut2 receptors and used to generate ATP. As the concentration of ATP in the cytosol increases, ATP-dependent potassium (K^+) channels close, preventing the outward flow of K^+ ions and causing the cell to depolarize. Voltage-gated calcium (Ca^{2+}) channels then open, allowing the influx of Ca^{2+}. Increasing cytosolic Ca^{2+} concentrations stimulate Ca^{2+} release from intracellular stores (such as the endoplasmic reticulum and the Golgi) and trigger the vesicular release of insulin.

via the common bile duct (Lacy and Kostianovsky, 1967; Gotoh et al., 1985), is now a widely used method for the preparation of high quality purified islets. Although they lack the vasculature and innervation found *in situ*, these isolated islets retain their glucose sensitivity and provide a valuable model system for *in vitro* investigations

In this thesis we used islets which were isolated from the pancreas by collagenase digestion and handpicked before being kept in culture. Experimental assays were performed on islets on the fourth day of culture as this was considered the optimal time to allow for viral-mediated genetic modulation to occur, while minimising the risk of changes in the innate calcium response to glucose which have been reported in cultured islets (Gilon et al., 1994).

3.2 ORGANOTYPIC CULTURES OF THE TUBEROINFUNDIBULAR DOPAMINE NEURONS

The TIDA neurons are a population of neuroendocrine, tyrosine hydroxylase (TH)-expressing cells (Fuxe, 1963; Pickel et al., 1975; Hokfelt et al., 1976; Chan-Palay et al., 1984; van den Pol et al., 1984) situated in the dorsomedial arcuate nucleus of the hypothalamus. They project their axons to the median eminence (Fuxe and Hokfelt, 1966; Lichtensteiger and Langemann, 1966) where they release dopamine into the portal circulation for transport to the anterior pituitary. Once in the pituitary, the dopamine exits the portal vasculature and acts on inhibitory dopamine D2 receptors on pituitary endocrine lactotroph cells to block them from releasing prolactin into the general circulation. During late pregnancy and in the immediate post-partum period, this dopaminergic blockade is lost, to allow for high circulating prolactin levels that drive milk production in the mother (Grattan, 2015). Recently the

electrophysiological properties of these neuroendocrine cells has been explored, revealing a robust oscillation (Lyons et al., 2010) which can be altered by neurotransmitters and hormones (Lyons et al., 2010; Lyons et al., 2012; Briffaud et al., 2015; Stagkourakis et al., 2016) to modulate dopamine release.

Ex vivo studies such as these use acute slice preparations which offer a snapshot of the network at the time of tissue extraction. However the relatively short lifespan of these slices is an obstacle to longer investigations, such as if genetic manipulation is desired. Since the 1980s organotypic slice cultures of neural tissue have become increasingly used as an alternative to *ex vivo* and cell culture techniques. Offering the advantages of being experimentally and visually more accessible than *in situ* approaches, longer lasting than acute slices and more physiologically relevant than cultured cells owing to the preservation of the tissue cytoarchitecture, organotypic cultures have been reported for various regions of the brain including the cerebellum, cortex, striatum, hippocampus and hypothalamus (Gahwiler, 1984; Bahr, 1995; Plenz and Kitai, 1996; House et al., 1998).

In this thesis we developed an organotypic slice culture model of the hypothalamic TIDA network. Due to the extensive characterisation of the electrophysiological properties of these neurons already reported (Stagkourakis et al., 2018), in addition to the fact that these cells express both NUCB1 and NUCB2 (Foo et al., 2010; Tulke et al., 2016), this neuroendocrine system was an ideal candidate for exploring the function of NUCB1 in neurons *in vitro*, allowing protein expression to be readily manipulated. DAT-Cre-tdTomato-floxed mice (Ekstrand et al., 2007) were used, to enable immediate visualisation of the TIDA neurons for electrophysiology procedures. The organotypic brain slice cultures were grown using the interface method, in which the extracted tissue is maintained on a semi-permeable membrane with medium below. In this arrangement only a thin film of medium covers the tissue, which allows adequate gas exchange while preventing the tissue from drying out (Stoppini et al., 1991; Fig. 4).

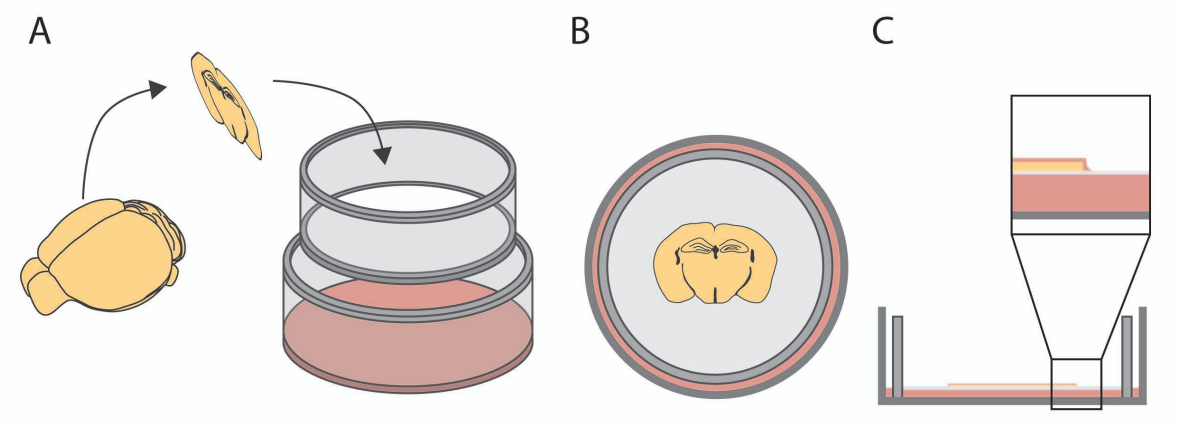

Figure 4: Schematic overview of the preparation of organotypic slices cultures. **A** The brain is dissected and coronal sections of the arcuate nucleus are cut and transferred to culture membrane inserts. **B** View from above showing the brain slice on the membrane in a 35 mm dish containing culture media. **C** Side view of the culture dish showing the media (pink) in contact with the membrane (white) but below the level of the brain slice (yellow). Inset highlights a thin film of media covering the brain slice by capillary action.

4 RESULTS AND DISCUSSION

4.1 DISTRIBUTION OF NUCB1 IN THE RODENT CNS

In the first study presented in this thesis we investigated the expression and distribution of NUCB1 in the CNS of the rat. The existence of NUCB2 in the brain has previously been demonstrated in discrete neuronal populations primarily centred in the hypothalamus (Oh et al., 2006; Brailoiu et al., 2007; Foo et al., 2008), yet a comparable exploration of the distribution of NUCB1 in the nervous system had not been reported.

Through the use of RT-PCR and *in situ* hybridisation, we identified a broad expression pattern of NUCB1 mRNA throughout the brain, which supported investigations at the protein level by immunofluorescence staining. Examination of all major brain regions including nuclei within the olfactory nucleus, cerebral cortex, striatum, hypothalamus and cerebellum, as well as the brain stem, the grey matter of the spinal cord and the dorsal root ganglia, failed to uncover a neuronal population that was devoid of NUCB1 staining. However, in spite of previous references to NUCB1 being a ubiquitously expressed protein (Lavoie et al., 2002; Leclerc et al., 2008; Kapoor et al., 2010; Kanuru et al., 2013), during these investigations it was soon apparent that not all cells are NUCB1-positive. Co-labelling experiments incorporating antibody markers for the widely used neuronal marker NeuN (Mullen et al., 1992) or the glial cell markers myelin basic protein (MBP; Kornguth and Anderson, 1965) and glial fibrillary acidic protein (GFAP; Bignami et al., 1972) revealed that the presence of NUCB1 was exclusive to neurons (Fig 5). Within these populations, there was no apparent preference for the expression of NUCB1 in any particular category of neuron, with the protein being equally conspicuous in, for example, interneurons *versus* projection neurons, or excitatory *versus* inhibitory neurons. This indicates that, unlike other well established calcium binding proteins such as parvalbumin and calbindin-D28K (Celio, 1990), which are expressed in anatomically and electrically distinct populations, NUCB1 is a general neuronal protein. Furthermore, while NeuN fails to identify a few types of neuron, such as the Purkinje and Golgi cells of the cerebellum, or the mitral cells of the olfactory bulb (Mullen et al., 1992), these cells were positively stained for

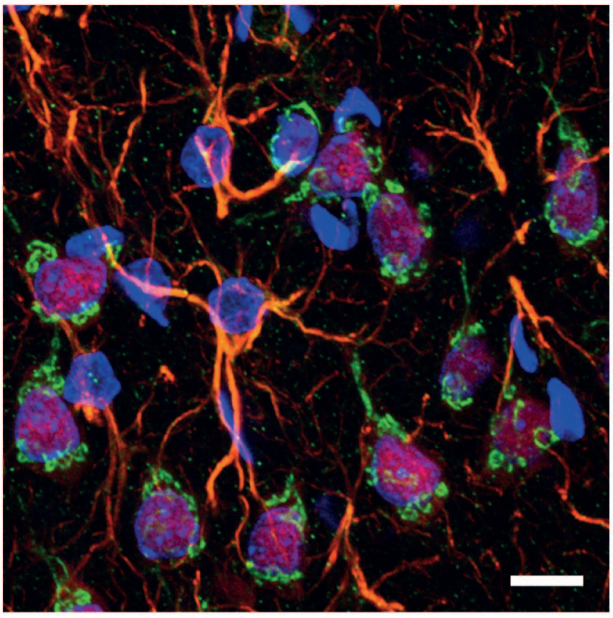

Figure 5: Immunofluorescent staining in the rat cortex for NUCB1 (green), NeuN (red) and GFAP (orange). Nuclei counterstained with DAPI (blue). Scale bar: 10 µm. *Tulke et al., 2016*

NUCB1. On this basis we propose that NUCB1 may serve as a more complete marker for the identification of neurons than NeuN.

4.2 EXPRESSION OF NUCB1 IN THE RODENT ENDOCRINE SYSTEM

The second study extended the work of Study I by investigating the distribution of NUCB1 in organs of the mouse endocrine system. Partly driven, again, by the lack of literature on this subject relative to the related protein NUCB2, whose expression had been reported in the pituitary (Foo et al., 2008; Stengel et al., 2009), gastrointestinal tract (Stengel et al., 2009; Zhang et al., 2010), pancreas (Gonzalez et al., 2009; Stengel et al., 2009; Foo et al., 2010) and testes (Stengel et al., 2009), we also wished to determine whether the broad but specific distribution of NUCB1 observed in neurons of the CNS was also true of the body's other major long-range signalling cell system.

Although primarily focused on the pancreas, we explored a range of other classical endocrine organs, namely the pituitary, gastrointestinal tract, adrenals, thyroid, parathyroid and gonads and, through RT-PCR and western blotting techniques, found that NUCB1 mRNA and protein was abundant in each of these tissues. Immunofluorescence studies again revealed that the distribution of NUCB1 was specific to subsets of cells within these tissues (*i.e.* not ubiquitous) and through histological and co-immunofluorescence analyses we identified these as constituting the endocrine components of the respective tissues. For example, in the pancreas the islets of Langerhans were positive for both NUCB1 mRNA and immunofluorescence, while the exocrine acinar cells displayed no such expression or labelling. Interestingly, one notable exception to this pattern of staining was the absence of NUCB1 detection in the interstitial tissue of the testes, where the testosterone-producing Leydig cells are located. This finding suggests that NUCB1 may not be integral to the operation of all secretory cells but rather only those of a particular type, for example, peptide-hormone secreting cells. Such a clear-cut distinction is unlikely given the presence of NUCB1 in the follicular cells of the thyroid, for example, but further investigation into the complete secretory repertoire of NUCB1-expressing cells could yield important insights into the function of the protein.

A common observation, underscored by colocalisation with the marker protein giantin (Linstedt and Hauri, 1993), in both the CNS and endocrine tissue studies was that the subcellular distribution of NUCB1 was restricted to the Golgi in all positive cells, supporting previous reports (Lin et al., 1998; Kawano et al., 2000; Lavoie et al., 2002). While primarily considered to be involved in the processing and packaging of proteins destined for secretion from the cell (Caro and Palade, 1961; Godman and Lane, 1964; Neutra and Leblond, 1966; Jamieson and Palade, 1967), there is now a growing appreciation of the Golgi as a key intracellular calcium store (Chandra et al., 1991; Pezzati et al., 1997) which participates in cellular signalling (Surroca and Wolff, 2000; Missiaen et al., 2004). Consequently, this location renders NUCB1 ideally situated to act as a sensor of calcium-mediated cell activation and participate in cell signalling.

Meanwhile, some studies have reported NUCB1 to be present in the nucleus, these observations were made in tumour (Wang et al., 1994; Kubota et al., 1998) or differentiating (Petersson et al., 2004) cells. Furthermore, the two former studies employed an antibody targeting an epitope in the N-terminal region of NUCB1, in contrast to the C-terminal-targeting one used in this thesis and by others. While the possible implications of this difference will be discussed in detail in section 4.4, it is important to note here that the immunofluorescence studies presented in this thesis were performed using one antiserum which may not represent the whole picture of NUCB1 distribution. However, the specificity of the antiserum in our tissue preparations was confirmed through pre-incubation with the corresponding immunogenic peptide, which selectively abolished the signalling pattern described (Fig. 6). In a further effort to support the specificity of the antibody, comparative immunofluorescence staining was performed using a commercial polyclonal antibody raised against the full length mouse NUCB1 protein. Although the signal intensity was notably weaker, the distribution of the protein at both the tissue and subcellular level appeared identical to that detected with the principal antisera.

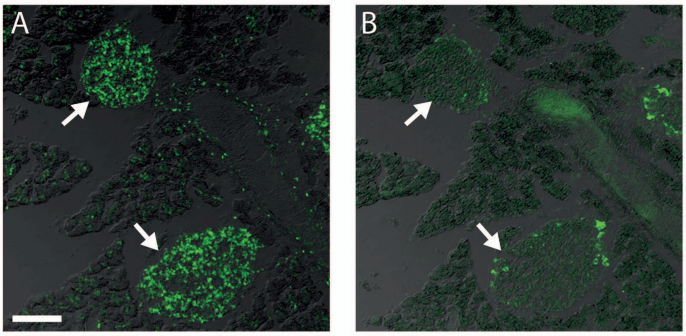

Figure 6: Specificity test of the NUCB1 antibody. Adjacent sections of mouse pancreas immunohistochemically stained with the antibody for NUCB1 (A) or the antibody for NUCB1 pre-incubated with the immunogenic peptide (B). Arrows denote the location of islets. Scale bar: 100 µm. *Williams et al., 2014*

In conclusion, Studies I and II demonstrate that NUCB1 is widely expressed throughout the nervous and endocrine systems, where its distribution appears to be specific to secretory cells and is consistently associated with the Golgi.

4.3 DEVELOPMENT OF AN ORGANOTYPIC SLICE CULTURE MODEL OF THE TIDA NETWORK

In the third study we developed and verified the physiological relevance of an organotypic slice culture model of the mouse TIDA network. Organotypic slice cultures provide a model in which living tissues can be maintained *in vitro* for a prolonged period of time while retaining their cytoarchitecture and cellular connectivity (Gahwiler, 1984). In order to determine whether an organotypic culture model of the TIDA network could function as a suitable model for studies complementary to those performed on acute slices, we examined the morphological and electrophysiological properties of mouse TIDA neurons in culture.

The TIDA neurons can be histochemically characterised by their expression of TH (Pickel et al., 1975; Hokfelt et al., 1976) and the dopamine transporter (DAT) (Meister and Elde, 1993) and we began by comparing the number of immunolabelled TH and genetically tagged DAT-tdTomato neurons in acute slices and cultures. After 13 days in vitro (DIV) we found a higher total number of marked cells in the organotypic cultures and, within this

population, a lower proportion of the cells expressed TH compared to the age matched P22 acute slices. While the organotypic slices are known to thin out over the first few days in culture (Stoppini et al., 1991), primarily as a result of cell death, it is not clear whether this cell death is a universal phenomenon predominantly driven by the cell injury experienced during brain slicing, or if certain neuronal populations are more susceptible to undergo apoptosis than others in the *in vitro* environment. As such, it remains a possibility that a greater number of TIDA/TIDA-progenitor cells are present in the 250 μm thick section of a P9 brain, compared to the same thickness in a P22 brain, and these cells are more resilient to the culture process, resulting in a greater number being present in the mature culture. A cell proliferation assay, such as bromodeoxyuridine (Gratzner, 1982; Miller and Nowakowski, 1988) or Ki-67 (Gerdes et al., 1984) immunolabelling and analysis of apoptosis through, for example, a TUNEL assay (Gorczyca et al., 1992) could yield very important information regarding the endurance of the TIDA cells as the tissue settles in culture. Furthermore, prolactin is known to regulate the expression of TH in TIDA neurons (Phelps et al., 1994; Arbogast and Voogt, 1995), as a means of homeostatic control, and therefore the lack of prolactin exposure in the organotypic cultures may be responsible for the proportionate reduction in TH positive cells. It would be of interest to maintain cultures in media supplemented with prolactin and determine whether this does indeed increase the expression of TH in the cultured TIDA neurons.

Irrespective of the increase in the number of TIDA neurons in the organotypic cultures, their morphology was comparable with their acute slice counterparts, with no difference seen in the arborisation of the dendrites. In terms of reconstructing the TIDA axons, however, cells in the organotypic cultures yielded significantly longer traceable projections. This is not surprising as the axons are less likely to be cut during slicing of the smaller P9 brains and they then have more restricted dimensions in which to develop once in culture, yet it highlights a distinct advantage of using the organotypic slice preparation for the study of axonal projections, for example investigating whether the TIDA neurons form synaptic connections with other neurons on their way towards the median eminence.

Analysis of the inherent electrophysiological properties of the TIDA neurons revealed that they are predominantly similar between the two preparations. A striking difference was, however, observed with regard to synaptic connectivity: a substantial increase in the amplitude and frequency of both excitatory (EPSCs) and inhibitory (IPSCs) postsynaptic currents. Interestingly a similar increase in miniature postsynaptic current frequency has been reported in CA1 hippocampal pyramidal neurons, which was linked to more extensive arborisation of the dendrites in culture (De Simoni et al., 2003). Although no such increase in dendritic length or arborisation was observed in the TIDA neurons, which inherently display far less prolific branching than pyramidal neurons, a change in spine density (not presently evaluated) could possibly account for this difference. Conversely, axonal projections were significantly longer in cultures and this may correlate with an increase in synaptic connectivity among TIDA cells, which have previously been shown to regulate their own network activity (Stagkourakis et al., 2016). This also raises the possibility that the increase in synaptic activity is a result of the TIDA neurons, or another cell population, being

relatively over-represented in the organotypic cultures. Alternatively, a lack of endogenous inhibition from other neuronal populations ordinarily present *in vivo* could impact on the tuning of the TIDA response to input. Equally feasible as an explanation to account for the increase in synaptic current amplitude is that the culture environment has permitted a change in receptor expression levels, altering the innate responsive properties of the cells. All of these possibilities warrant further investigation to elucidate which factors dictate how the TIDA neurons develop in culture compared to in the intact brain.

In general, however, the morphological and electrophysiological properties of the TIDA network in organotypic culture are comparable to the acute slice. We conclude that this preparation provides a viable model not only for further investigations into the TIDA network, but also into the general properties of neural and neuroendocrine systems.

4.4 INVESTIGATIONS INTO THE FUNCTION OF NUCB1

In the fourth study presented in this thesis we investigated the possible function of NUCB1. Although previous research into NUCB1 has explored aspects of the protein's functional domains, few, if any, of these studies have addressed the overarching potential of a protein capable of binding both DNA and calcium. Indeed the DNA-binding capacity for which the protein was originally named appears to have been largely forgotten in the pursuit of ascribing a function to its calcium-handling properties. In this study we set out to ascertain whether NUCB1 plays a role in the regulation of DNA transcription or calcium signalling within cells of the nervous and endocrine system, employing the isolated islets of Langerhans and the organotypic culture of the TIDA network as representative models.

We first explored whether NUCB1 could be stimulated to translocate to the nucleus. The mapping studies presented in this thesis (Studies I and II) found NUCB1 to be localised to the Golgi, corresponding to previous reports (Lin et al., 1998; Kawano et al., 2000; Lavoie et al., 2002). However, the structure of the protein contains a DNA-binding motif (Miura et al., 1992) and a nuclear localisation signal (Wendel et al., 1995) while some studies have already reported it in the nucleus (Wang et al., 1994; Kubota et al., 1998; Petersson et al., 2004; Somogyi et al., 2004). We therefore hypothesised that NUCB1 may, under certain conditions, translocate to the nucleus and bind to DNA. Calcium is known to mediate glucose-stimulated insulin secretion (Grapengiesser et al., 1988; Santos et al., 1991) and this could also be the trigger for the activation and mobilisation of NUCB1. However, incubating isolated islets in a high concentration of glucose did not alter the location of NUCB1. An alternative stressor to islets is the state of pregnancy, during which beta cell proliferation is high (Parsons et al., 1992; Rieck et al., 2009). In the islets of pregnant mice at gestation day 14-15, at which point the rate of beta cell proliferation is at its peak (Rieck and Kaestner, 2010), NUCB1 remained detectable only in association with the Golgi. As mentioned earlier, these localisation studies were performed using one antiserum raised against a C-terminal fragment of NUCB1. It is possible that this C-terminal epitope is exposed when NUCB1 is in its Golgi-associated conformation yet, in the process of moving to the nucleus, its conformation changes, hiding the epitope and thus rendering the protein undetectable with this antibody. That NUCB1 changes conformation upon binding calcium has been demonstrated previously (de Alba and

Tjandra, 2004), as has its presence in the nucleus in studies using an antibody raised against a fragment from the N-terminal region of the protein (Wang et al., 1994; Kubota et al., 1998). On this basis it would be premature to conclude from this study that NUCB1 does not translocate to the nucleus, but rather highlights that an alternative approach could be required to identify such a phenomenon, for example obtaining an effective antibody raised against another region of the protein for comparative immunofluorescence staining, or overexpressing NUCB1 with a conjugated fluorescent tag to enable live image tracking of the protein's intracellular movements.

We also investigated whether modulation of NUCB1 expression has an impact on insulin expression. Insulin mRNA levels were unaffected in islets in which NUCB1 had been either knocked down or overexpressed, indicating that NUCB1 is not directly involved in the regulation of insulin transcription. We then turned our attention to the calcium binding capacity of NUCB1 by examining the effect of modulated levels of the protein on calcium handling in the beta cell. Upon stimulation of islets with 11 mM glucose, cytosolic calcium oscillations were observed in accordance with those previously described (Grapengiesser et al., 1988), with no differences detected between treatments. This was also reflected in the insulin secretion capacity of the islets, with ELISA measurements revealing that insulin release was neither attenuated nor augmented by altered NUCB1 levels. Calcium imaging was performed using the cytosolic reporter dye Fura-2. As the Golgi has been shown to contribute to stimulation-coupled intracellular calcium release (Surroca and Wolff, 2000; Missiaen et al., 2004), it was hypothesised that alterations in the expression of NUCB1 may impact on calcium movement within the cell as a whole. However, as the endoplasmic reticulum provides a larger source of intracellular calcium, any anomalies in the Golgi release may be easily compensated for by this organelle. As NUCB1 is typically localised to the Golgi lumen (Lin et al., 1998), it would therefore be more advantageous to look specifically at calcium handling in the Golgi by, for example, using a calcium indicator genetically targeted to the Golgi lumen (Missiaen et al., 2004; Lissandron et al., 2010).

Finally, we investigated the properties of TIDA neurons in which NUCB1 had been knocked down. Here we found that reduced levels of NUCB1 led to a decrease in the frequency of spontaneous EPSCs and IPSCs. There was no concurrent change in spine density or post synaptic density protein 95 expression, suggesting that NUCB1 may not impact the anatomical organisation of the network, but rather influence the intracellular signalling cascade of the cells. It remains to be determined whether this could be through, for example, regulation of membrane receptor expression or moderation of calcium signalling, but the findings presented here warrant further investigations into the role of NUCB1 in cellular communication.

These studies involving modulation of NUCB1 expression were performed using adenoviral delivery of an shRNA vector under the control of the U6 promoter or NUCB1 overexpression linked to the rat insulin promoter (RIP). It is therefore important to note that while overexpression of NUCB1 occurred specifically in the pancreatic beta cells, knockdown of the protein was not specifically targeted and would have occurred in the other islet and neuronal cell types. Intra-islet signalling plays an important part in the regulation of

hormone secretion with, for example somatostatin release from delta cells known to influence alpha and beta cell activity (Taborsky et al., 1979; Schuit et al., 1989). For this reason the effects (or lack of) of NUCB1 knockdown in the islets cannot be taken as conclusive at this time. Similarly, the change in TIDA neuron post synaptic currents observed following NUCB1 knockdown must be interpreted with caution as the activity of surrounding non-TIDA neurons would likely have been equally modified. A more rigorous approach would involve specifically targeting these cells, such as through the use of Cre-dependent viral targeting of the TIDA neurons or beta cells.

The results of this study indicate that NUCB1 does not play a direct role in regulating the synthesis or secretion of hormones, but may function to modulate signalling responses in secretory cells. Further studies should be performed to elucidate the precise mechanisms by which post synaptic cell activity is influenced by the expression of NUCB1.

5 CONCLUSION

The work presented in this thesis set out to investigate the distribution and function of the DNA- and calcium-binding protein NUCB1. In these studies we demonstrate that the protein is widely expressed in secretory cells throughout the rodent central nervous and endocrine systems, where it is ordinarily localised to the Golgi. Using a well-established model of an endocrine cell system, the islets of Langerhans, and a newly developed model of the neuroendocrine hypothalamic TIDA neurons, we explored the possible function of NUCB1 in secretory cells. While we found no evidence of its participation in the regulation of secretory factor synthesis or release, we propose that it may be involved in the mediation of cell communication, possibly influencing the signal cascades which lead to stimulated secretion.

The data presented here set the foundation for further studies into the role of this intriguing protein. The beta cell and TIDA neuron models described in this thesis offer suitable systems for additional experimental investigations, yet consequent findings could pertain to a more general understanding of the operation of NUCB1 in secretory cells throughout the body.

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